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The Role of Thrombocytes in Liver Fibrogenesis: Effects of Platelet Lysate and Thrombocyte-Derived Growth Factors on the Mitogenic Activity and Glycosaminoglycan Synthesis of Cultured Rat Liver Fat Storing Cells

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Summary: A central problem in the study of the pathogenesis of liver fibrosis (fibrogenesis) is the identification of the cellular sources of the extracellular matrix and the dissection of the molecular mediators stimulating connective tissue synthesis in certain hepatic target cells. In the present study the role of platelets and of some platelet-derived polypeptide growth factors in the proliferation and proteoglycan synthesis of rat liver fat storing cells in culture (the principle connective tissue-producing cell type in liver) was determined. Fat storing cell proliferation was determined by measurement of the DNA-content, and [^{3}H]thymidine- and bromodeoxyuridine-incorporation. Glycosaminoglycan synthesis was determined by the measurement of [^{35}S]sulphate incorporation.

Human platelet lysate (0.3 to 2.6 g protein per litre medium) stimulated, in a dose-dependent manner, both the proliferation and glycosaminoglycan synthesis of rat liver fat storing cells kept as a primary culture in *Dulbecco*'s modification of *Eagle*'s medium in the absence of foetal calf serum. More than 70% of the newly synthesized glycosaminoglycans were found in the medium. Among the various thrombocyte-derived polypeptides tested as candidate mediators of the platelet-derived fibrogenic activity, platelet derived growth factor was not effective in enhancing glycosaminoglycan synthesis, and it stimulated the proliferation of fat storing cells only about 2 fold. On the other hand, epidermal growth factor proved to be a stimulus of both processes. Transforming growth factor β (> 10 pmol/l) inhibited foetal calf serum (*Dulbecco*'s modification of *Eagle*'s medium with a fraction of foetal calf serum of 0.1) and epidermal growth factor stimulated proliferation but enhanced the synthesis of sulphated glycosaminoglycans about 2-fold. These results suggest the possible role of transforming growth factor β as a negative modulator for fat storing cells proliferation but a positive modulator for fat storing cell transformation and extracellular glycosaminoglycan matrix synthesis. Furthermore, our results indicate a cooperation between different hepatic and extrahepatic cell types by paracrine stimulation of fat storing cells. Transforming growth factor β in combination with epidermal growth factor appear to be candidate mediators of the platelet-derived fibrogenic activity, which stimulates fat storing cells in culture, and might also be effective in vivo during hepatic repair processes following liver injury.

Introduction

Persistent liver cell injury, irrespective of its aetiology, may be followed by liver fibrosis, which is defined by

(i) an excess and irregular deposition of connective tissue elements in the intercellular space,

(ii) a molecular rearrangement of connective tissue composition, and

(iii) modifications of the fine structure of the various connective tissue molecules (1).

The main components of human and animal liver extracellular matrix are several types of collagens (2–5), some structural glycoproteins (fibronectin, laminin) (6), a group of proteoglycans (proteoheparan sulphate, proteochondroitin sulphate, proteodermatan (sulphate) (1, 7–9), the glycosaminoglycan, hyaluronic acid (7, 10), and elastin. In fibrotic liver the concentration of all extracellular matrix elements, except heparan sulphate, is elevated several-fold. The cells responsible for producing the various connective tissue components are hepatocytes, which synthesize predominantly heparan sulphate, as well as fibronectin, laminin (6) and potentially some collagens (2, 3) and certain non-parenchymal liver cells (fat storing cells, endothelial cells), which produce several extracellular matrix components. Liver fat storing cells attract increasing interest, because under certain conditions fat storing cells proliferate strongly (4, 9, 11, 12) and transform into myofibroblast like cells (13). These stain positively for the cytoskeletal protein, desmin (14), and they produce significant quantities of connective tissue components and, interestingly, a pattern of glycosaminoglycans and collagen similar to that found in fibrotic liver extracellular matrix (1, 4, 5, 7, 8, 15, 16). These reactions are considered to be important events in human and animal liver fibrogenesis (1, 5, 7, 10, 16–18). So far, little is known about the factors (fibrogenic mediators) which promote proliferation/transformation and extracellular matrix synthesis of fat storing cells. Recently, studies from others and from our laboratory have shown that secretions of activated *Kupffer* cells and monocytes stimulated the proliferation/transformation of fat storing cells in culture (9, 12), as well as stimulating the synthesis of proteoglycans (16) and hyaluronic acid (10) by these cells. Activated macrophages produce platelet derived growth factor, transforming growth factor α (19) and transforming growth factor β, all of which may be candidate mediators of macrophage-derived fibrogenic actitiviy. The findings that

(i) an enhanced pool of platelets together with infiltrated inflammatory cells are seen by electron microscopy in liver cell necrotic areas (20),

(ii) platelets are the richest source of transforming growth factor β1 (21, 22), and

(iii) platelet derived growth factor and the transforming growth factor α-like epidermal growth factor may be released from alpha granules of platelets following platelet activation (23), support the hypothesis that thrombocytes might be involved in liver fibrogenesis by stimulating the growth, transformation and extracellular matrix synthesis of fat storing cells.

The purpose of this study was to isolate and cultivate rat liver fat storing cells, in order to study the stimulatory potency of human thrombocyte lysate and of platelet derived growth factor, epidermal growth factor and transforming growth factor β on fat storing cell proliferation/transformation and on glycosaminoglycan synthesis. Our results indicate that

(i) thrombocytes stimulate fat storing cell proliferation and the synthesis and secretion of sulphated glycosaminoglycans by these cells,

(ii) transforming growth factor β and epidermal growth factor are probably effector molecules of platelet-derived fibrogenic activity.

Materials and Methods

Materials

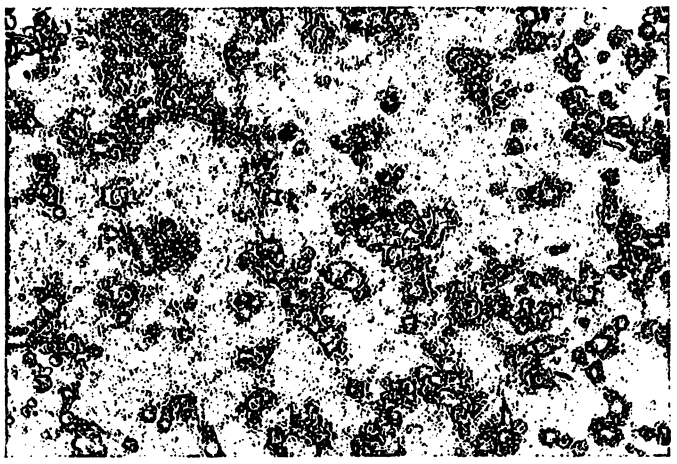
Thrombocyte concentrate was isolated by centrifugation at 300 *g* (1500 min^{-1}) (Fenwal CS-3000 cell separator) from normal human donor blood. Human epidermal growth factor (β-urogastrone), collagenase H (clostridiopeptidase A, EC 3.4.24.3) and foetal calf serum were from Boehringer Mannheim, FRG. Transforming growth factor β (source human platelets) was purchased from ICN Biochemicals, Cleveland, Ohio, USA. Monoclonal mouse antibodies to BrdUrd and nuclease were from Amersham Buchler, Braunschweig, FRG. [^{3}H]thymidine (673 TBq/mol) and [^{35}S]sulphate (18.5–22.2 TBq/mol) were from New England Nuclear Corp., Boston, Mass. USA and Nycodenz (analytical grade) was from Nyegaard and Co. AS, Oslo, Norway. Calf thymus DNA (type I), pronase and platelet derived growth factor (source human platelets) were from Sigma Chemical Company, Munich, FRG. Peroxidase-conjugated rabbit-anti-mouse-immunoglobulins and antibodies to desmin and vimentin were purchased from Dakopatts, Glostrup, Denmark.

Preparation of thrombocyte lysate

Human thrombocyte concentrate (kindly supplied by Prof. *Kretschmer*, Abt. f. Transfusionsmedizin, Universitätsklinik Marburg) was lysed by sonification, centrifuged at 4 °C for 20 min at 3000 *g* and the supernatant was dialysed extensively (cut off M_r 3500) against *Dulbecco*'s modification of *Eagle*'s medium at 4 °C.

Cell isolation and cell culture

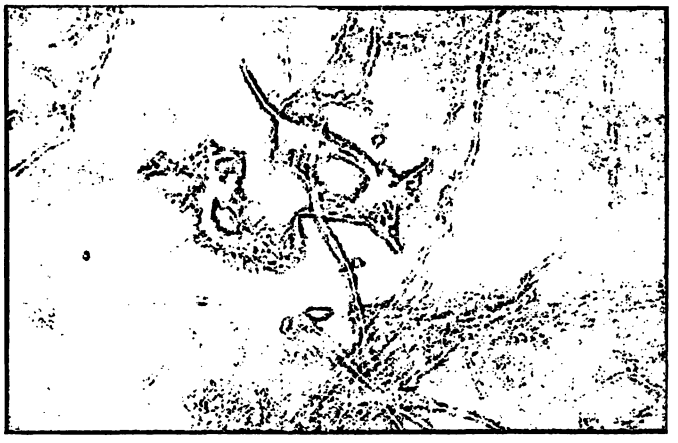
Detailed procedures for the isolation and culture of fat storing cells from rats have been published previously from this laboratory (7–9, 16). Briefly, the cells were isolated from one year old, 500–650 g male Sprague Dawley rats (Lippische Versuchstierzucht, Extertal, FRG). Non-parenchymal cells were isolated by the pronase/collagenase method of de *Leeuw* et al. (4), as originally described by *Knook* et al. (24), with some minor modifications described in detail elsewhere (8). They were purified by a single-step Nycodenz density gradient (82 g/l). The cells were seeded at a density of 0.2–0.4 × 10^6 cells/2 cm^2 on 24-well culture plates (Greiner, Nürtingen, FRG), or at a density of 0.4 × 10^6 cells/10 cm^2 on 6-well culture plates (Greiner, Nürtingen, FRG), or at a density of 0.04–0.1 × 10^6 cells/cm^2 on 1 cm^2 glass cover slips. They were then grown in *Dulbecco*'s modification of *Eagle*'s medium (Flow Laboratories GmbH, Bonn, FRG) with 4 mmol/l *L*-glutamine, 100 kU/l penicillin,



a)

d)

b)



e)

c)

Fig. 1. Microscopy of rat liver fat storing cells in primary culture.

a) Light microscopy 18 h after seeding (before 2nd medium change). Seeding density was 0.02 × 10^6 cells/cm^2. Fat storing cells show numerous lipid droplets arround the nucleus. Magnification 75×.

b) Phase contrast microscopy of a single fat storing cell on the 5th day of culture. The cell is flat and shows long cellular extensions and residues of cytoplasmic lipid droplets. Magnification 175×.

c) Indirect immunoperoxidase staining for desmin. Primary culture of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum 8 days after seeding. About 70% of the cells are stained. Examples of desmin-negative cells are indicated by an arrow. Seeding density was 0.02 × 10^6 cells/cm^2. Magnification 75×.

d) Phase contrast microscopy of the same cells as in fig. 1c. Examples of desmin-negative cells are indicated by an arrow. Magnification 75×.

e) Indirect immunofluorescence staining for vimentin. Primary culture of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium with a fraction of 0.1 foetal calf serum 6 days after seeding. Many fibrils of vimentin are recognized in the cytoplasm. Magnification 160×.

100 mg/l streptomycin and a fraction of 0.1 foetal calf serum in a humidified atmosphere of 0.05 CO_2 and 0.95 air at 37 °C. The purity of the fat storing cell preparations was assessed by light-microscopy (fig. 1a), using the presence of perinuclear lipid droplets and the typical cell shape as markers, and by vitamin A-specific autofluorescence (8). At a later stage of the culture, purity was assessed by phase contrast microscopy (fig. 1b) and by immunofluorescence/immunoperoxidase staining for desmin (fig. 1c) and vimentin (fig. 1d). The mean purity of freshly isolated cells was greater than 85%, and cell viability assessed by trypan blue exclusion was more than 80%. With the first (about 8 h after seeding) and second (about 20 h after seeding) medium change, most of the contaminating cells were removed, and the fat storing cell monolayers were essentially free from impurities. With the third medium change (about 44 h after seeding) foetal calf serum in *Dulbecco*'s modification of *Eagle*'s medium was omitted and thrombocyte lysate (5—40 µl = 0.325—2.6 mg protein) was added. In experiments to study the effects of platelet-derived growth factors, the fractional content of foetal calf serum was decreased to 0.005 at the third medium change, and test substances [platelet derived growth factor (0.25—5 µg/l), epidermal growth factor (5—50 µg/l), and transforming growth factor β (0.025—6 µg/l)] were added. Twenty four hours later, the medium was changed in order to introduce radiolabelled precursors, and this new medium also contained fresh supplements of growth factors.

Determination of cell proliferation

DNA measurement

The DNA content of the fat storing cell monolayer (4 days after seeding) was measured fluorometrically using calf thymus DNA (type I, Sigma Chem. Co., Munich, FRG) as a standard (25).

[^{3}H]Thymidine incorporation

Thymidine incorporation was measured after trichloroacetic acid precipitation of cells, which had been exposed for 24 h to [^{3}H]thymidine (74 MBq/l medium), beginning on the third day after seeding (9).

Bromodeoxyuridine incorporation

To study BrdUrd incorporation, fat storing cells were seeded at a density of 0.04—0.1 × 10^6 cells/cm^2 in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum on 1 cm^2 glass cover slips in 6 well (3 ml/well) macroplates (Greiner, Nürtingen, FRG). Twenty four h after seeding, foetal calf serum was decreased to 5 ml/l medium, and growth factors were added. Eighteen h before the end of the culture, BrdUrd (50 µmol/l final concentration) was added. At the end of the culture period, monolayers were washed twice with phosphate buffered saline-*Dulbecco*'s modification of *Eagle*'s medium (pH 7.4) and fixed with ethanol/acetic acid 95 + 5 (by vol.). DNA was then denatured by incubating the cells for 45 min at 37 °C with BrdUrd antibodies and nuclease. After washing 3 times with phosphate buffered saline (pH 7.4) cells were incubated with peroxidase-conjugated rabbit anti-mouse-immunoglobulins for 45 min at 37 °C. Cells were then washed again 3 times with phosphate buffered saline, and the nuclei, which had taken up BrdUrd, were stained with 3,3′-diaminobenzidine tetrahydrochloride as substrate and hydrogen peroxide 3%, nickel chloride and cobalt chloride 30 g/l in H_2O as intensifier.

Determination of the synthesis of total glycosaminoglycans

The synthesis of sulphated glycosaminoglycans was determined from the incorporation of [^{35}S]sulphate (18.5—22.2 TBq/mol; 740 MBq/l medium) during a labelling period of 24 h [details described elsewhere (7, 8, 16)]. Usually, the radionuclide was added after a medium change on the 3rd day of primary culture. Cultures were stopped by removing the medium, washing the cell layer three times with phosphate buffered saline and freezing the cells. The cells were detached from the well by repeated (3 ×) freezing and thawing and scraped off with a pipette tip. Less than 1% of the radioactivity remained in the well. Details of the procedure to determine the synthesis and secretion of sulphated glycosaminoglycans are described elsewhere (8). Briefly, glycosaminoglycans were isolated by binding to DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.3 mol/l sodium acetate buffer (pH 6.6), extensively washed with the same buffer, and thereafter eluted with 2.2 mol/l NaCl. An aliquot of the eluate was mixed with Instagel (Packard Instruments Company, Downers Grove, Ill., USA) and counted for radioactivity. Glycosaminoglycan synthesis was expressed as radioactivity per mg DNA. In experiments using thrombocyte lysate to stimulate fat storing cells, glycosaminoglycans were measured separately in the medium and cell-layer of the cultures. Glycosaminoglycans of the cell layer were determined after proteolysis with papain (EC 3.4.22.2) as described previously (8).

Results

Fat storing cell proliferation

Effect of thrombocyte lysate

Proliferation of fat storing cells in foetal calf serum-free medium was stimulated by thrombocyte lysate in a dose dependent manner (fig. 2). [^{3}H]thymidine incorporation and DNA content per culture were increased to a maximum of 8- and 2-fold, respectively, 48 h after the addition of 40 µl (2.6 mg protein) thrombocyte lysate to primary cultures of fat storing cells grown on 10 cm^2 wells in 2 ml *Dulbecco*'s mod-

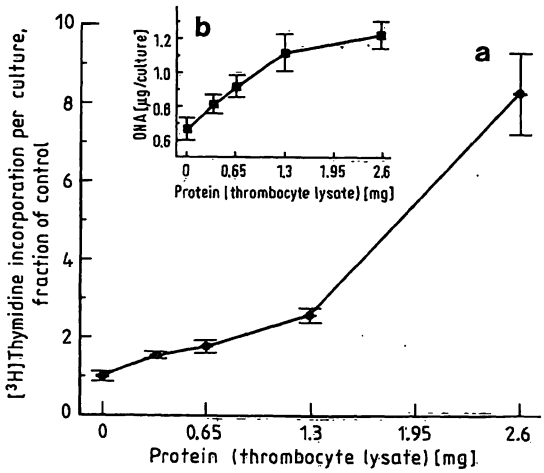


Fig. 2. Proliferation of fat storing cells measured by incorporation of [^{3}H]thymidine into DNA (a) and DNA content per culture (b). Cells were labelled with [^{3}H]thymidine (74 MBq/l) for 24 h between the 3rd and 4th culture day.
Thrombocyte lysate (0.65—2.6 mg protein/culture) was added on the second and third day after seeding. Cells were seeded at a density of 0.1 × 10^6 cells/cm^2. Control culture received *Dulbecco*'s modification of *Eagle*'s medium without foetal calf serum. Mean values ± SD of three independent experiments, each with n = 4.

ification of *Eagle*'s medium without foetal calf serum. If fat storing cells were cultured in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum, the addition of thrombocyte lysate caused only a 2-fold stimulation of [^{3}H]thymidine incorporation and it had no significant effect on the DNA content.

Epidermal growth factor-stimulated fat storing cell proliferation

Incubation of subconfluent rat fat storing cells with human epidermal growth factor in the presence of 5 ml/l foetal calf serum resulted in a dose-dependent stimulation of cell proliferation, as measured by [^{3}H]thymidine incorporation (fig. 3a), DNA content per culture well (fig. 3b) and BrdUrd incorporation (tab. 1). Twenty µg/l (3.3 nmol/l) epidermal growth factor stimulated [^{3}H]thymidine incorporation to a maximum of 3.5-fold, compared with controls. The EC_{50} for epidermal growth factor-stimulated [^{3}H]thymidine incorporation was calculated to be about 8 µg/l (1.3 nmol/l) using 7 concentrations of epidermal growth factor ranging from 5 to 1000 µg/l. Fifty µg/l epidermal growth factor produced maximal DNA synthesis (fig. 3b). In the presence of a higher foetal calf serum concentration (100 ml/l), epidermal growth factor failed to stimulate fat storing cell proliferation further (data not shown).

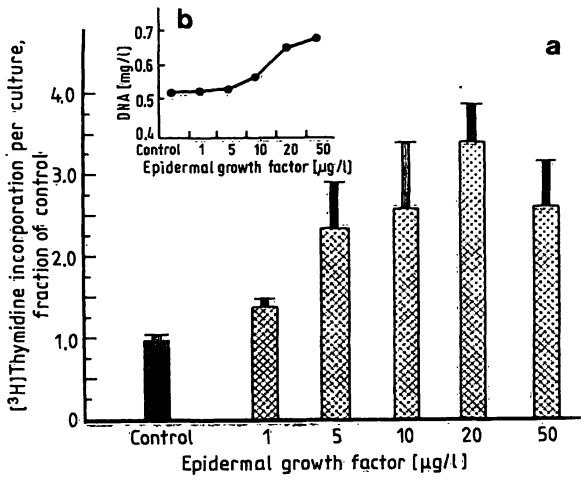


Fig. 3. Dose-dependent stimulation of fat storing cell proliferation measured by [^{3}H]thymidine incorporation into DNA (a) and DNA content (b) by human epidermal growth factor. Cells were seeded at a density of 0.06 × 10^6 cells/cm^2 in 6-well culture plates and grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum. Human epidermal growth factor (1–50 µg/l) was added on the second and third day after seeding.
Between the 3rd and 4th culture day cells were labelled with [^{3}H]thymidine 74 MBq/l medium for 24 h. Control culture was kept in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum. Mean values ± SD of three independent experiments, each with n = 4.

Tab. 1. Effect of foetal calf serum (FCS) and epidermal growth factor (EGF) on fat storing cell proliferation.
Proliferation was measured by bromodeoxyuridine incorporation. Cells were seeded at a density of 0.06 × 10^6 cells/cm^2 on glass cover slips in *Dulbecco*'s modification of *Eagle*'s medium (DMEM) containing a fraction of 0.1 foetal calf serum. After 24 h the fraction of foetal calf serum was reduced to 0.005, and epidermal growth factor (5 µg/l or 50 µg/l) was added. Eighteen h before the end of the culture cells were labelled with BrdUrd (5 × 10^{-5} mol/l final concentration). Nuclei which had taken up BrdUrd were stained by immunoperoxidase method using antibodies to BrdUrd. The control represents *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum (0.5% FCS).

Immunoperoxidase (anti-BrdUrd)-stained nuclei (fraction of total cells)

Control (0.005 FCS)	DMEM + 0.10 FCS	EGF (5 µg/l)	EGF (50 µg/l)
0.045	0.2	0.07	0.18

Effect of transforming growth factor β on fat storing cell proliferation and transformation

Transforming growth factor β (0.025–2.5 µg/l, 1–100 pmol/l) inhibited, in a dose dependent manner, the proliferation of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 or 0.1 foetal calf serum (fig. 4). Since

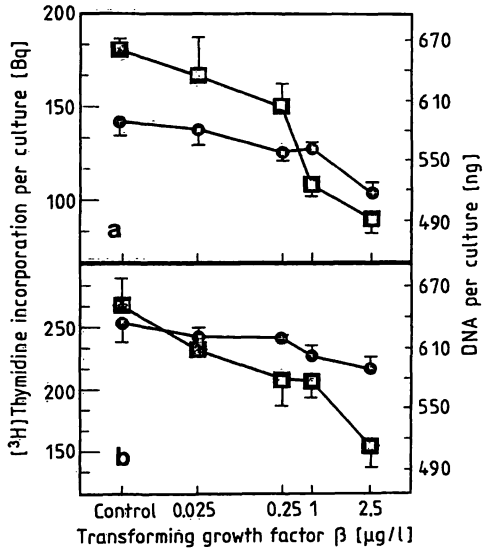


Fig. 4. Transforming growth factor β-induced dose-dependent inhibition of fat storing cell proliferation measured by [^{3}H]thymidine incorporation into DNA (■–■) and DNA content (●–●). Cells were seeded at a density of 0.06 × 10^6 cells/cm^2 in 6-well culture plates and grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum [a] or in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum [b]. Transforming growth factor β was added on the 2nd and 3rd day after seeding. Between the 3rd and 4th culture day cells were exposed for 24 h to [^{3}H]thymidine 74 MBq/l medium. Values represent mean ± SD of 4 cultures.

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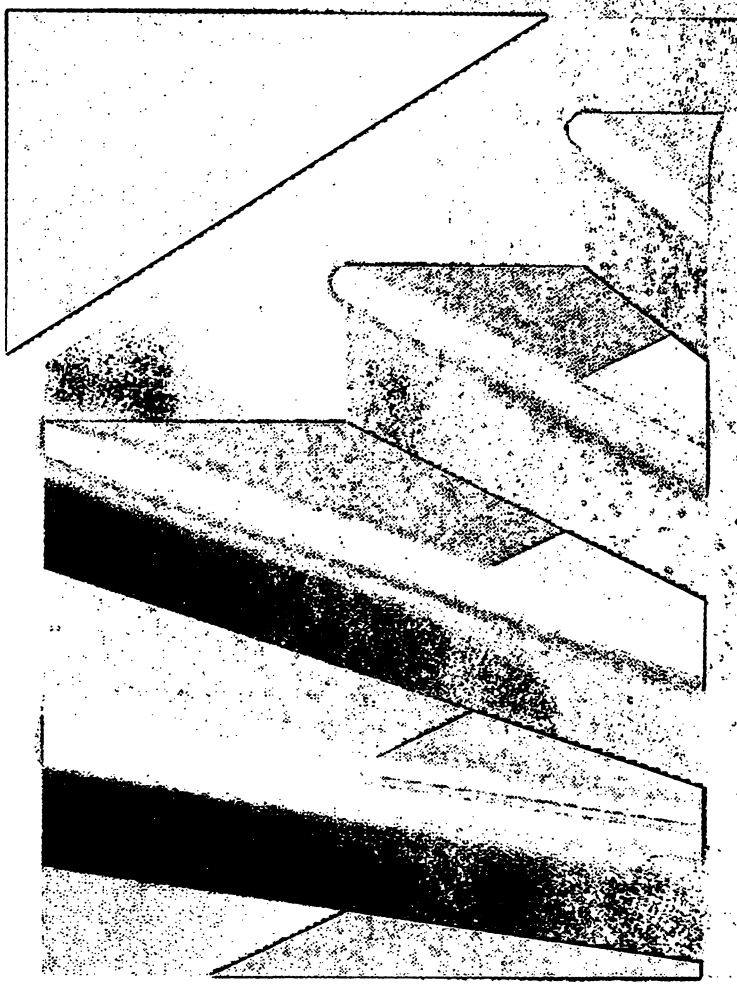

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the culture period was only 4 days, the inhibition of [^{3}H]thymidine incorporation was more pronounced (significant reduction with 10 pmol/l transforming growth factor β) than the decrease of the DNA content (significant reduction with 100 pmol/l transforming growth factor β). A higher transforming growth factor β concentration (6 µg/l, 240 pmol/l), added on second and third day after seeding to primary cultures of rat fat storing cells (containing 0.005 foetal calf serum) inhibited basal [^{3}H]thymidine incorporation by 90%, while epidermal growth factor (50 µg/l, 9.1 nmol/l) stimulated cell proliferation by 92% (fig. 5). In a further experiment, fat storing cells were seeded at a density of 3000 cells/cm^2 and grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum in the presence or absence of 20 pmol/l transforming growth factor β. Cultures were stopped after 2, 6, 8, 14, 16 and 18 days. Total cell numbers were counted using phase-contrast microscopy, and the fraction of desmin-positive cells was counted after immunoperoxidase staining for desmin. While inhibiting cell growth, transforming growth factor β stimulated fat storing cell transformation to desmin-positive myofibroblast-like cells (fig. 6).

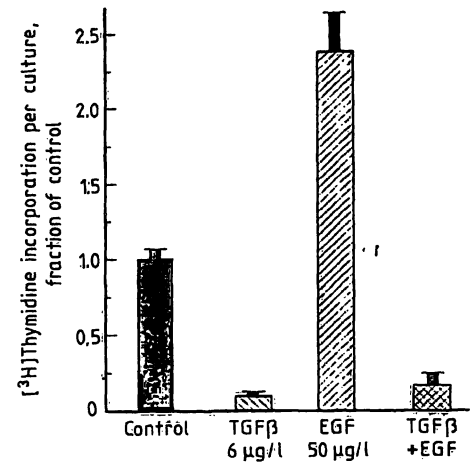

Fig. 5. Effect of transforming growth factor β (TGFβ) on epidermal growth factor (EGF) stimulated fat storing cell proliferation measured by [^{3}H]thymidine incorporation. Cells were seeded at a density of 0.06 × 10^6 cells/cm^2 in 6 well culture plates and grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum. The controls were cultures kept in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum. Transforming growth factor β was added simultanously with epidermal growth factor on the 2nd and 3rd day of culture. Cells were exposed for 24 h to [^{3}H]thymidine 74 MBq/l medium.

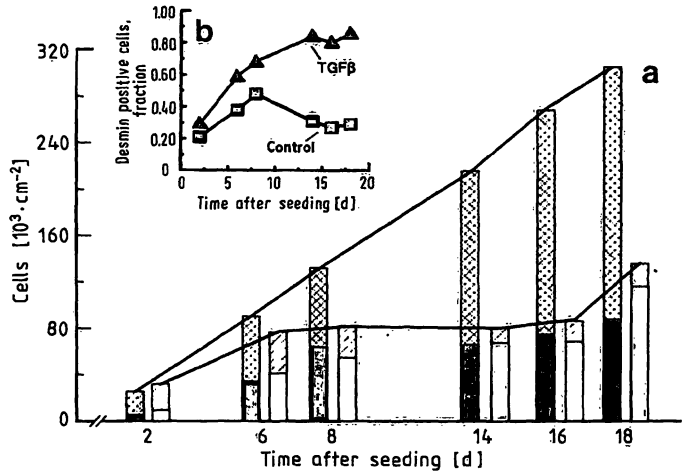

Fig. 6. Effect of transforming growth factor β on fat storing cell growth and transformation to desmin-positive myofibroblast-like cells. Cells were seeded at a density of 3 × 10^3 cells/cm^2 and grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum in the presence [▨] and absence [▩] of transforming growth factor β. Transforming growth factor β (20 pmol/l) was added on the 1st, 5th, 7th, 13th, 15th and 17th day of culture. Cultures were stopped on the 2nd, 6th, 8th, 14th, 16th and 18th day after seeding. In fig. 6a the black bars represent the number of desmin-positive cells per cm^2 in control, the grey bars represent the number of desmin-positive cells per cm^2 in transforming growth factor β-treated cultures. The inset b) demonstrates the relative amount (fraction of total cells) of desmin-positive cells in control (■—■) and in transforming growth factor β-treated cultures (▲—▲). Total cell numbers were counted using phase-contrast microscopy (see fig. 1d) and desmin-positive cells were counted using light microscopy after immunoperoxidase staining for desmin (see fig. 1c). Mean values of 2 representative experiments are given.

Platelet derived growth factor-stimulated fat storing cell proliferation

Platelet derived growth factor (0.5 µg/l, 16.6 pmol/l) was added, on the 2nd day after seeding to primary cultures of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium without foetal calf serum. Under these conditions, platelet derived growth factor stimulated fat storing cells proliferation, as measured by [^{3}H]thymidine incorporation, to a maximum of 2.1-fold compared with controls (tab. 2). The DNA content was increased by platelet derived growth factor (2 µg/l, 66 pmol/l) during 48 h of stimulation to 1.24 fold of control.

Synthesis and secretion of glycosaminoglycans by rat liver fat storing cells

Stimulation by thrombocyte lysate

Thrombocyte lysate caused a dose-dependent increase (up to 8-fold) of the incorporation of [^{35}S]sulphate (per mg DNA) into the total proteoglycans of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium without foetal calf serum (fig. 7). There was a preferential incorporation of [^{35}S]sulphate into the proteoglycans of the medium (about 11-fold), whereas proteoglycan radioactivity associated with the cell layer increased only 4-fold in the presence of lysate. When fat storing cells were cultured in *Dul-*

Tab. 2. Effect of platelet-derived growth factor on fat storing cell proliferation measured by [^{3}H]thymidine incorporation. Cells were seeded at a density of 0.1 × 10^6 cells/cm^2 in 6-well culture plates and kept in *Dulbecco*'s modification of *Eagle*'s medium without foetal calf serum (FCS). Different concentrations of platelet derived growth factor (0.25–5 µg/l) were added on the 2nd and 3rd day of culture. Between the 3rd and 4th day cells were exposed for 24 h to [^{3}H]thymidine 74 MBq/l medium. The mean values ± SD of 4 cultures are shown.

[^{3}H]thymidine incorporation (Bq/culture)				
Control	Platelet-derived growth factor [µg/l]			
without FCS	0.25	0.5	2	5
11.7 ± 2.1	20.6 ± 4.8	24.5 ± 1.4	18.3 ± 2.3	19.6 ± 1.5

becco's modification of *Eagle*'s medium containing a fraction of 0.1 foetal calf serum, the stimulatory effect of thrombocyte lysate on proteoglycan synthesis was much weaker (only 2-fold), suggesting that the enhancement of proteoglycan synthesis by foetal calf serum is largely due to platelet derived factors.

Epidermal growth factor-stimulated glycosaminoglycan synthesis

In the presence of 5 ml/l foetal calf serum, epidermal growth factor significantly stimulated proteoglycan synthesis, as measured by [^{35}S]sulphate incorporation in the proteoglycans of the medium. Epidermal growth factor (3.3 nmol/l, 20 µg/l), stimulated [^{35}S]sulphate incorporation per culture 2.3-fold, and per mg DNA 1.6-fold, compared with controls.

Transforming growth factor β-stimulated glycosaminoglycan synthesis

Treatment of fat storing cells with transforming growth factor β caused a dose-dependent enhanced radiosulphate incorporation into secreted (medium) glycosaminoglycans, with a maximal increase of about 2-fold at 2.5 µg/l in *Dulbecco*'s modification of *Eagle*'s medium containing 5 ml/l foetal calf serum (fig. 8a) and a maximal increase of about 1.7 fold at 1 µg transforming growth factor β per litre *Dulbecco*'s modification of *Eagle*'s medium containing 100 ml/l foetal calf serum (fig. 8b). In a preliminary experiment we found that in controls (*Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum) most (75%) of the incorporated [^{35}S]sulphate was present in the dermatan-sulphate fraction, whereas heparan-sulphate contained 20% and chondroitin-sulphate only 5% of the radiosul-

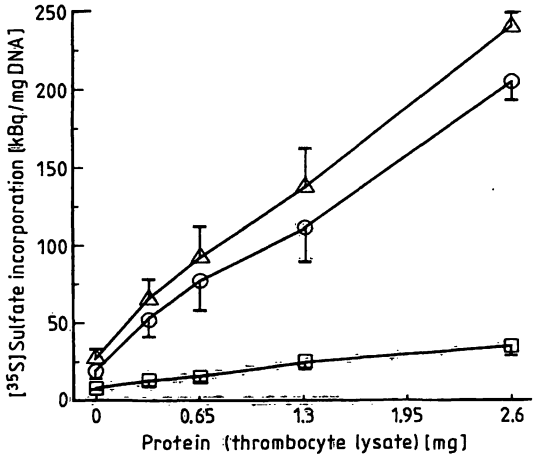

Fig. 7. Effect of thrombocyte lysate on [^{35}S]sulphate incorporation into glycosaminoglycans of cell layer and medium. Thrombocyte lysate (0.65–2.6 mg protein/culture) was added on the second and on the third day after seeding. Cells were seeded at a density of 0.1 × 10^6 cells/cm^2. Control cultures received *Dulbecco*'s modification of *Eagle*'s medium without foetal calf serum. After labelling for 24 h with [^{35}S]sulphate (740 MBq/l medium) glycosaminoglycans of cell layer (□–□) and medium (○–○) were analysed separately. Values are expressed as mean ± SD of three independent experiments, each with n = 4 on the basis of DNA content. △–△ = total radioactivity.

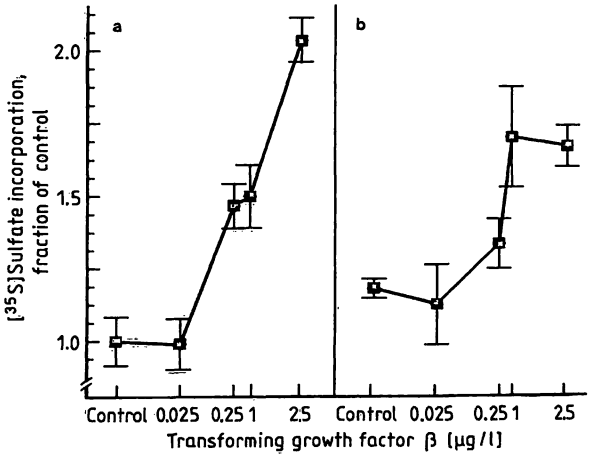

Fig. 8. Incorporation of [^{35}S]sulphate into medium glycosaminoglycans of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 (a) or 0.1 foetal calf serum (b), respectively. Cells were seeded at a density of 0.06 × 10^6 cells/cm^2 in 6-well culture plates. Transforming growth factor β was added on the 2nd and 3rd day after seeding. Between the 3rd and 4th culture day cells were exposed for 24 h to 740 MBq [^{35}S]sulphate per litre medium. Values are expressed as mean ± SD of 4 cultures on the basis of DNA.

phate. Addition of transforming growth factor β reduced the relative radiosulphate content of dermatan-sulphate from 75% to 62%, and of heparan-sulphate from 20 to 10%, but increased the relative [^{35}S]sulphate content of chondroitin-sulphate from 5 to 32%. In contrast to newly synthesized and secreted dermatan-sulphate and heparan-sulphate, the absolute [^{35}S]sulphate incorporation, expressed as radiosulphate incorporation per mg DNA into chondroitin-sulphate, was also significantly stimulated by transforming growth factor β (at 0.5 µg/l about 7-fold and at 2.0 µg/l more than 8-fold) compared with controls.

Effect of platelet derived growth factor on glycosaminoglycan synthesis

Platelet derived growth factor (0.25 – 5.0 µg/l, 8.3 – 166 pmol/l), added on the 2nd day after seeding to primary cultures of fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium without or with a fraction of 0.1 foetal calf serum, did not significantly increase the quantity of glycosaminoglycan synthesised per mg DNA.

Discussion

The ability of liver fat storing cells to proliferate strongly (9, 11, 12), and to transform into myofibroblast-like cells (4, 11, 13) which produce significant quantities of connective tissue components, is considered to be an important event in human and animal liver fibrogenesis (1, 5, 7, 10, 16, 17, 18). Following all types of hepatic injury, an infiltration of the tissue with blood monocytes occurs, and this is paralleled by an enhanced proliferation of *Kupffer* cells (26) followed by a stimulated proliferation and transformation of fat storing cells. These observations and the electron microscopic demonstration of platelets together with infiltrated inflammatory cells in liver cell necrotic areas (20) support the hypothesis that factors released from these cells might directly promote the growth, transformation and extracellular matrix synthesis of fat storing cells.

Studies from others and from our laboratory support this hypothesis. It was shown recently that secretions of activated *Kupffer* cells and monocytes stimulate the proliferation/transformation of fat storing cells in culture (9, 12), as well as the synthesis of proteoglycans (16) and hyaluronic acid (10) by these cells. Comparing the magnitude of matrix proteoglycan and hyaluronate synthesis by fat storing cells with that of other liver cells, it was concluded that the fat storing cell is the most important cell in liver fibrogenesis (7). In cultures of fat storing cells labelled for 24 h with [^{3}H]glucosamine or [^{35}S]sulphate, more than 70% of the labelled glycosaminoglycans are found in the extracellular medium (7). At present, the function of the sulphated glycosaminoglycans and other matrix elements secreted by fat storing cells is poorly understood. Several authors propose that the interaction of cells with the extracellular matrix influences cell behaviour, affecting cell adhesion and motility, growth and differentiation (for review see l. c. (27)). Important new aspects of the putative functions of glycosaminoglycans are provided by observations showing that type III transforming growth factor β receptor is a membrane heparan/chondroitin sulphate proteoglycan (28, 29) and that certain glycosaminoglycans act as reservoirs of growth factors (30). However, we have so far been unable to relate the high output of glycosaminoglycans by fat storing cells to specific autocrine or paracrine effects.

In the present study we demonstrated for the first time that platelets contain fibrogenic mediators which stimulate not only proliferation, but also the synthesis and secretion of glycosaminoglycans into the extracellular matrix by fat storing cells in primary culture. The main growth factors elaborated by platelets are transforming growth factor β (platelets are the richest source of transforming growth factor β1 (21)), platelet derived growth factor (31) and epidermal growth factor (23). The data presented in this study clearly demonstrate that primary cultures of rat fat storing cells grown in *Dulbecco*'s modification of *Eagle*'s medium containing a fraction of 0.005 foetal calf serum show a mitogenic response to human epidermal growth factor and platelet derived growth factor. Recently, we demonstrated that the epidermal growth factor-like transforming growth factor α, which is produced by activated macrophages (19) and probably *Kupffer* cells (*Bachem*, unpublished data), binds to epidermal growth factor receptor on fat storing cells with a similar affinity to that of epidermal growth factor, and stimulates the proliferation of fat storing cells in culture (32). Epidermal growth factor and transforming growth factor α are equally potent in the stimulation of fat storing cell proliferation.

Transforming growth factor β, a M_r = 25000 disulphide-linked homodimer originally found in transformed fibroblasts (21, 22) belongs to a family of growth factors that regulate cell growth and differentiation (for review see l. c. (33)). Transforming growth factor β has been found to be both a stimulator (33, 34) and an inhibitor of cell growth (28, 33 – 37) depending on the system studied. We showed that transforming growth factor β strongly inhibited the proliferation of fat storing cells stimulated by foetal

calf serum or epidermal growth factor. Similar results, i.e. a dose-dependent inhibition of the epidermal growth factor-stimulated proliferation, were obtained with cultured rat aortic smooth muscle cells (37). As proposed by *Sporn* et al. (38) and *Roberts* et al. (34) transforming growth factor β stimulates or inhibits proliferation as a function of the entire set of growth factors present within the cell. Several in vitro studies indicate that transforming growth factor β may control the effects of other polypeptide growth factors, such as platelet derived growth factor, epidermal growth factor/transforming growth factor α, basic fibroblast growth factor and interleukin 2, and that transforming growth factor β can determine whether a cell responds in a positive or negative manner to such a growth factor (for review see l.c. (38)). This action of transforming growth factor β seems to be exerted via modulation of growth factor receptors, since it was shown recently that transforming growth factor β inhibits epidermal growth factor/transforming growth factor α binding to the high affinity epidermal growth factor cell surface receptors on fibroblasts (39), and furthermore that the platelet derived growth factor-stimulated proliferation of bone marrow fibroblasts is also inhibited by interaction of transforming growth factor β with platelet derived growth factor binding (35).

In cultured normal human dermal fibroblasts transforming growth factor β stimulates the synthesis of types I and III collagens (40, 41) and fibronectin (40, 41, 42). In endothelial cells, fibronectin synthesis is also stimulated by transforming growth factor β (36). It is suggested that transforming growth factor β may play a role in the normal regulation of extracellular matrix production in vivo, and may contribute to the development of pathological states of skin (41) and organ (43) fibrosis. The observation by *Wiseman* and coworkers (44), that transforming growth factor β at very low concentrations (16–100 ng/l) may recruit monocytes from the circulation and activate them to express factors like tumour necrosis factor α, may also by very relevant to fibrogenesis. Transforming growth factor β stimulates collagen synthesis in fat storing cells (45). We show here for the first time that transforming growth factor β added to primary cultures of fat storing cells stimulates glycosaminoglycan synthesis and extracellular deposition of glycosaminoglycans. In bovine cartilage organ cultures, transforming growth factor β was also shown to regulate the metabolism of glycosaminoglycans (46). Preliminary results showing that the synthesis of chondroitin sulphate is preferentially stimulated by transforming growth factor β agree with observations made by others in mouse mammary epithelial cells (27).

Our results demonstrate that thrombocytes release fibrogenic mediators which stimulate fat storing cell proliferation/transformation and glycosaminoglycan synthesis by these cells. All three polypeptide growth factors studied (epidermal growth factor, platelet derived growth factor and transforming growth factor β) are candidate mediators of platelet-derived fibrogenic activity in culture, and in vivo during hepatic injury. Others have shown that in acute CCl_4 liver damage the level of transforming growth factor β mRNA rises 24 h later and peaks at 48 h (47), and that transforming growth factor β gene expression is significantly enhanced during active fibrogenesis associated with liver disease in man (48). Together with these observations, the results presented here indicate a central, fundamental role for transforming growth factor β in hepatic fibrogenesis.

Conclusion

The observation that activated *Kupffer* cells, monocytes and thrombocytes, whose numbers are increased in areas of liver cell injury, stimulate fat storing cell proliferation/transformation and extracellular matrix synthesis, indicates the existence of cooperative interactions between different hepatic and extrahepatic cell types, mediated by paracrine mechanisms. These interactions seem to be important in liver fibrogenesis, a process in which transforming growth factor β may play a central role.

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